bio-protocol Preprint

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ISH and IHC

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Updated date: Aug 5, 2022

An abbreviated version of this protocol was published in eLIFE in Nov 2021

Single-nucleus transcriptomic analysis of human dorsal root ganglion neurons

DOI: 10.7554/eLife.71752

Detailed protocol

ISH signal intensity from human DRGs is highly dependent on the quality of the tissue. For some DRGs, even highly expressed genes yield weak signal intensity. So, we focused only on those DRGs that gave us strong signals for both weak and high expressing genes. We also noticed that the longer the tissues/sections have been stored at -80^{o}C, the weaker the signal. Even in DRGs where we initially obtained strong signal, we observed signal degradation after the sections were stored at -80^{o}C for more than several months.

HiPlex ISH was performed using a custom HiPlex12 assay from Advanced Cell Diagnostics (ACD), following the manufacturer's instructions. The current HiPlex12 equivalent kit available from ACD is the HiPlex12 Reagent Kit v2 (488, 550, 650) (cat# 324419).

Below is a brief protocol summary. The protocol from ACD can be found here:
https://acdbio.com/sites/default/files/UM%20324419%20RNAscope%20HiPlex%20v2%20User%20Manual%20%28488%2C%20550%2C%20650%29.pdf

Cryosections from human DRGs were cut at 20 µm and stored at -80^{o}C until use.

Fix sections:

1. Fix with 4% PFA at RT for 60 mins. (Remove slides from -80^{o}C and immediately immerse in 4% PFA at RT)
2. Wash in 1x PBS (move slides up and down 3-5 times or with gentle agitation of slide mailer)

Dehydrate sections:

1. 50% EtOH – 5 min at RT
2. 70% EtOH – 5 min at RT
3. 100% EtOH – 5 min at RT
4. 100% EtOH – 5 min at RT

Optional – store slides in 100% EtOH at -20^{o}C for up to 1 week

Create hydrophobic barrier:

1. Remove slide from 100% EtOH and air dry for 5 min on clean paper towel
2. Use Immedge hydrophobic barrier pen (Vectorlabs) to draw barrier 2-4 times
3. Let barrier dry for 1 min

Apply Protease IV

1. Add protease IV to cover section
2. Incubate for 30 min at RT
3. Remove protease IV and wash in 1x PBS with slight agitation
4. Repeat 1x PBS wash (slides should not stay in 1x PBS for longer than 5 min)

Prepare probes and reagents

1. Warm HiPlex probe stocks (50X) and HiPlex diluent at 40^{o}C for 10 min
2. Dilute probes to 1x. (Mixed probes can be stored at 2-8^{o}C for up to six months)
3. Place HiPlex Amp 1-3 and HiPlex Fluoro T1-T3 reagents at RT

Hybridize probe

1. After 1x PBS wash, add mixed probes to section
2. Incubate for 2 hrs at 40^{o}C
3. Wash 2x with 1x wash buffer for 2 min each at RT

Amplification 1-3

1. Add HiPlex Amp 1 to section
2. Incubate 30 min at 40^{o}C
3. Wash 2x with 1x wash buffer for 2 min each at RT

1. Add HiPlex Amp 2 to section
2. Incubate 30 min at 40^{o}C
3. Wash 2x with 1x wash buffer for 2 min each at RT

1. Add HiPlex Amp 3 to section
2. Incubate 30 min at 40^{o}C
3. Wash 2x with 1x wash buffer for 2 min each at RT

HiPlex Fluoro T1-T3

1. Add HiPlex Fluoro T1-T3 to section
2. Incubate 15 min at 40^{o}C
3. Wash 2x with 1x wash buffer for 2 min each at RT
4. Counterstain with DAPI for 30 sec at RT
5. Remove DAPI
6. Add 1-2 drops of ProLong Gold Antifade Mountant (ThermoFischer Scientific, cat# P36930)
7. Add coverslip and seal with Cytobond removable coverslip sealant (SciGene, cat # 2020-00-1) (slides can be stored in the dark at 2-8^{o}C for up to 3 days)
8. Image

Cleave fluorophores

1. Soak slide in 4x SSC at RT for at least 30 min to remove coverslip sealant and coverslip
2. Wash the slide once in 4x SSC
3. Break open a fresh glass ampoule of cleaving stock solution
4. Prepare a 10% cleaving solution by diluting with 4X SSC
5. Add 10% cleaving solution to section
6. Incubate 15 min at RT
7. Wash slide 2x with PBST (0.5% Tween) for 2 min each

Repeat cleavage (steps 5-7)

1. Add 10% cleaving solution to section
2. Incubate 15 min at RT
3. Wash slide 2x with PBST (0.5% Tween) for 2 min each

HiPlex Fluoro T4-T6

1. Add HiPlex Fluoro T4-T6 to section
2. Incubate 15 min at 40^{o}C
3. Wash 2x with 1x wash buffer at RT for 2 min each

Mount slides

1. Add 1-2 drops of ProLong Gold Antifade Mountant and seal slide with Cytobond removable coverslip sealant
2. Image round 2

Cleave fluorophores

1. Soak slides in 4x SSC at RT for at least 30 min to remove coverslip sealant and coverslip
2. Wash the slide once in 4x SSC
3. Break open a fresh glass ampoule of cleaving stock solution
4. Prepare a 10% cleaving solution by diluting with 4X SSC
5. Add 10% cleaving solution to section
6. Incubate 15 min at RT

6. Incubate 15 min at RT
7. Wash slide 2x with PBST (0.5% Tween) for 2 min each

Repeat cleavage (steps 5-7)

1. Add 10% cleaving solution to section
2. Incubate 15 min at RT
3. Wash slide 2x with PBST (0.5% Tween) for 2 min each

HiPlex Fluoro T7-T9

1. Add HiPlex Fluoro T7-T9 to section
2. Incubate 15 min at 40^{o}C
3. Wash 2x with 1x wash buffer at RT for 2 min each

Mount slides

1. Add 1-2 drops of ProLong Gold Antifade Mountant and seal slide with Cytobond removable coverslip sealant
2. Image round 3

Cleave fluorophores

1. Soak slides in 4x SSC at RT for at least 30 min to remove coverslip sealant and coverslip
2. Wash the slide once in 4x SSC
3. Break open a fresh glass ampoule of cleaving stock solution
4. Prepare a 10% cleaving solution by diluting with 4X SSC
5. Add 10% cleaving solution to section
6. Incubate 15 min at RT
7. Wash slide 2x with PBST (0.5% Tween) for 2 min each

Repeat cleavage (steps 5-7)

1. Add 10% cleaving solution to section
2. Incubate 15 min at RT
3. Wash slide 2x with PBST (0.5% Tween) for 2 min each

HiPlex Fluoro T10-T12

1. Add HiPlex T10-T12 to section
2. Incubate 15 min at 40^{o}C
3. Wash 2x with 1x wash buffer at RT for 2 min each
4. Counterstain with DAPI for 30 sec at RT
5. Remove DAPI and add ProLong Gold Antifade Mountant seal slide with Cytobond removable coverslip sealant
6. Image round 4

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Nguyen, M. , Ryba, N. and Davidson, S. (2022). ISH and IHC. Bio-protocol Preprint. bio-protocol.org/prep1845.
2. Nguyen, M. Q., von Buchholtz, L. J., Reker, A. N., Ryba, N. J. and Davidson, S.(2021). Single-nucleus transcriptomic analysis of human dorsal root ganglion neurons. eLIFE. DOI: 10.7554/eLife.71752